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PRINCIPAL INVESTIGATOR: Yun-Fai Chris Lau, Ph.D.

CONTRACTING ORGANIZATION: Northern California Institute for Research and Education
San Francisco, California 94121

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Yun-Fai Chris Lau, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Northern California Institute for Research and Education
San Francisco, California 94121

E-Mail: clau@itsa.ucsf.edu

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13. ABSTRACT *(Maximum 200 Words)*

Currently information regarding the contribution of the men-only chromosome, the Y chromosome, to prostate cancer is lacking. The goals of this project are designed to address this question and to identify candidate genes on the Y chromosome involved in prostate cancer. The objectives are: 1) to study the expression of Y chromosome genes in prostate cancer and 2) to evaluate their effects in over-expression in the prostate of transgenic mice. So far, we have completed a survey on the expression of 31 Y chromosome genes in prostate cancer and have identified TSPY gene to be the most likely one to play a role in male oncogenesis. In Year 2, we have isolated and sequenced several polymorphic TSPY transcripts in normal and cancerous prostate samples, and demonstrated that over-expression of TSPY potentiates cell proliferation in vitro and tumor formation in vivo. In Year 3, we plan to address if the various TSPY isoforms have the same function in cell proliferation and to test the hypothesis on whether over-expression of TSPY in the prostate is sufficient in promoting oncogenesis in this organ using transgenic mouse strategies. These studies will provide important information on the role of this male-specific chromosome on prostatic oncogenesis and in development of new diagnostic procedures, and/or treatment for prostate cancer.

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INDRODUCTION

The Y chromosome is the male-specific chromosome in the human genome. It plays critical roles in not only switching on a male developmental pathway during embryogenesis but also serves vital functions in male-specific organs, such as testis and prostate glands. Its role in oncogenesis of these male-specific organs, including cancers of the prostate and testis, is still undefined. Previous studies had demonstrated either a gain or a loss of this chromosome in cytogenetic preparations of cancer samples, leading to the postulations of an oncogenic and tumor suppressing role respectively for this male chromosome. More recent investigations, particularly those conducted under the present research project, had now demonstrated the potential oncogenic properties of some genes on this chromosome in both prostate and testis cancers.

There are two specific objectives for the present project. The first one is designed to identify the most likely candidate oncogene among the genes on this chromosome using a series of expression techniques. The second one is to evaluate the roles of the identified candidate(s) in oncogenic activities in transgenic mice, particularly on the prostate glands. These results will be compared to those of known oncogenes, such as SV40 T antigen and/or c-Myc, expressed in the prostate (1). These studies should provide valuable information on the potential role of the human Y chromosome in prostatic oncogenesis.

BODY

Task 1. To determine the expression patterns and probable functions of the Y chromosome genes in prostate cancer.

Through a series of expression studies conducted on all 31 genes on the human Y chromosome, at the initial phase of this project, we have identified the repeated gene, testis-specific protein Y or TSPY, to be a significant candidate that potentially has a role in prostatic oncogenesis (2). This gene is differentially expressed in various prostate cancer samples and is inducible with androgen in the prostatic cancer cell line, LNCaP. TSPY has been mapped to a region of the human Y chromosome postulated to harbor a cancer predisposition gene, termed gonadoblastoma locus on the Y chromosome or GBY. Gonadoblastoma develops with high incidence (>30%) in XY sex-reversed individuals or patients with Turner syndrome harboring some Y chromosome materials (3). These patients have either a non-functional or a deleted male-determining gene, SRY, and hence develop into females, despite the presence of part or the entire Y chromosome in their genome. Deletion mapping has identified a small region of the Y chromosome containing the TSPY gene and predisposing these patients to gonadoblastoma development. Expression studies had indeed demonstrated a TSPY expression in tumor tissues of gonadoblastoma (4). Further, TSPY was also detected at high levels in testicular cancer of germ cell origins. These results suggested that TSPY is a putative candidate for GBY. Further studies, conducted under the present research project, demonstrated that TSPY is indeed expressed in prostate cancers of various degrees of malignancy. These studies further supported a role for TSPY in prostatic oncogenesis. Because of the successful completion of the proposed studies under Task 1, we have proposed to further investigate the TSPY gene in oncogenesis, as outlined in the Year 1 Progress Report.

PROPERTIES OF THE TSPY GENE

The TSPY gene was independently isolated in my laboratory (5) and that of Jorge Schmidtke in Germany (6). Early studies demonstrated that the predominant transcripts were derived from transcriptional units of 2.8 kb in size consisting of 6 exons and 5 introns (5,7). TSPY has been

postulated to serve a normal function in directing the spermatogonial cells to enter meiosis (3,7). Significantly, it shares tight protein homology to the SET oncogene, initially identified in a chromosome 9 rearrangement in a patient suffering from undifferentiated leukemia (8,9). The SET oncogene encodes a protein belonging to a family of cyclin B-binding proteins (10,11). Binding analysis conducted in our laboratory has indeed demonstrated the cyclin-B binding property of TSPY protein (data not shown), supporting the notion that TSPY is another member of this protein family that includes the nucleosome binding proteins (NAP1, NPL1). These proteins bind to mitotic cyclin B and contribute to the complex mechanisms of cell cycle regulation (9,11,12). Hence inappropriate expression of TSPY in either a female gonad (as in the case of gonadoblastoma) or the prostate gland (which normally expresses only a low level of TSPY) may be responsible for predisposition to and/or initiation of oncogenesis in these tissues. This hypothesis suggests that TSPY may function either as an oncogene or as an oncogenic promoter in both testicular and prostate cancers.

EVIDENCE SUPPORTING THE EXISTENCE OF POLYMORPHIC TSPY PROTEINS

Studies in both our laboratory and that of Jorge Schmidtke demonstrated three specific types of transcripts from the TSPY genes (5,7,13). The first one is a predominant transcript that encodes 309 amino acids. This transcript is designated as type 1 TSPY transcript. The cDNA initially isolated in our laboratory was derived from a transcript utilizing an alternate acceptor site, 11 nucleotides ahead of that at exon 6 for the type 1 mRNA, resulting in a protein with 295 amino acids. This minor transcript is designated as type 2 mRNA for TSPY. A variant type 1 transcript consists of an additional 18-nucleotide repeat within the first exon that is in-frame with the coding sequence, resulting in a protein with 315 amino acids. This rare transcript is designated as type 3 mRNA of the TSPY gene. All three types of transcript encode slightly polymorphic TSPY proteins that harbor a conserved (NAP) domain homologous to that shared by the cyclin B binding proteins.

To determine if other forms of TSPY transcript may exist in normal and diseased tissues, we have recently examined the transcript population of TSPY in normal and cancerous samples of the testis and prostate gland. The entire coding sequences of different TSPY transcripts were amplified by PCR with specific primers from cDNAs synthesized from total RNAs of respective samples, cloned in plasmid vector and sequenced completely in both directions using an automated sequencer. The resulting sequences were then analyzed with the sequence analysis computer program, MacVector, and the BLAT program at the Genome Center, University of California, Santa Cruz. The BLAT program identifies the structural genes, including intron, exon and splice junctions, of the transcriptional units from the draft sequences of the entire human genome. Results from this study demonstrated a complex array of splice variants of TSPY transcripts in both prostatic and testis samples. They can be classified into two categories. The first one concerns the first exon in which a cryptic donor site at sequence immediately following amino acid residue #29 is used to splice into three different acceptor sites within exon 1 and exon 2. The first two variants splice into sequence preceding amino residue #118 and #135 respectively in exon 1 while the third one splices into that preceding amino residue #170 in exon 2. These in-frame cryptic RNA splices result in the deletion of 93, 110 and 145 amino acids from their respective ORFs. These transcripts are designated as variant Exon1A, Exon1B and Exon1C respectively. The splice variants on exon 1 are primarily type 1 transcripts. The second category of variant transcripts involves the skipping of the small introns, 3 and/or 4, in the RNA processing, resulting in altered reading frames beyond the additional sequences and slightly different proteins at the carboxyl ends. All encoded proteins, however, harbor the NAP domain in their respective ORFs. Specific primers flanking these new splice junctions had been used in RT-PCR analysis to confirm the presence of splice variants in both testis and prostate samples (data not shown). Although various forms of TSPY transcripts were present in both testis and prostatic samples, the shortened versions (e.g. Exon1A, Exon1B and Exon1C) of type 1 transcript were relatively higher in the prostates than the testis

samples. This observation could be significant if the respective TSPY isoforms serve different/variable biological functions.

Task 2. Functional evaluation of Y chromosome genes in prostate cancer.

Initially we proposed to use a transgenic mouse strategy to test the oncogenic roles of Y chromosome genes identified to potentially play a role in prostatic oncogenesis. Our identification of TSPY gene as a significant candidate for the GBY gene on the Y chromosome and its likely role in prostate cancer have necessitated a refinement of our evaluation strategy, as outlined in the Year 1 Progress Report. We have adopted a strategy to address the probable mechanism of TSPY action in cell cycle modulation and its potential contribution to oncogenesis. Our working hypothesis is that TSPY is a specialized cell cycle regulator for male spermatogonial cells in the testis and when it is aberrantly expressed in male-specific organs, such as prostate and testis, it causes abnormal cell proliferation, leading to oncogenesis. To address this hypothesis, we used a cell culture system to determine the function of an over-expression of TSPY in cell proliferation using the tet-off system (as originally proposed). Once determined to have an influence in cell proliferation, an in vivo tumorigenicity assay using athymic nude mice was used to evaluate the consequence of such an over-expression of the TSPY gene.

OVER-EXPRESSION OF TSPY POTENTIATES CELL PROLIFERATION

The presence of the NAP domain in all TSPY variant proteins clearly suggests that this domain is very important for the biological function of this Y chromosome gene. As discussed above, many of the cyclin B binding proteins are involved in cell cycle regulation. It is conceivable, therefore, that TSPY may interact with the mitotic cyclin in spermatogonial cells and exert it function in mediating these sperm stem cells in entering meiosis. When it is aberrantly expressed in the prostate, it may potentiate a somatic cell proliferation, contributing to the multi-step oncogenic process. To examine this possibility, we have utilized the tet-off transgene regulation system (14) to manipulate the expression of a transfected TSPY gene in cultured HeLa and NIH3T3 cells. In this system, both HeLa and NIH3T3 cells harbor a constitutively expressed transactivator gene that can transactivate a responder gene, consisting of responsive promoter and a bicistronic construct coding for both TSPY and a tracer green fluorescent protein (EGFP). In the presence of tetracycline or its derivative doxycycline, such transactivation is abolished, repressing the TSPY and EGFP expression. Hence, one can compare the effects of over-expression of the target genes between two identical cell populations cultured in the absence or presence of the antibiotic.

Several independent cell clones were isolated from stable transfection of HeLa or NIH3T3 tet-off cells using either the bicistronic construct, TIG-TSPY, or the corresponding vector, TIG, alone. Cells transfected with a functional TIG-TSPY construct and cultured in selective media without doxycycline gave a consistently higher number of colonies than those selected with media containing doxycycline or transfected with vector alone. Expression analyses of both EGFP (direct observation) and TSPY (immunofluorescence) demonstrated that both proteins were co-expressed in the same cells (data not shown). Their expression could be tightly regulated by doxycycline. Cell proliferation was analyzed using the XTT kit (Roche Biochemicals) that measures the enzyme activity of the mitochondrial succinate tetrazolium reductase, considered to be proportional to the number of metabolically active cells. Results from these analyses demonstrated that the proliferative activities of both HeLa and NIH3T3 cells increase to about 30-40% of the respective parental cells or cells transfected with vector alone. Such increases were abolished by addition of doxycycline in the media, resulting in repression of the responder gene in the cells. Hence, over-expression of TSPY potentiates cell proliferation in vitro.

OVER-EXPRESSION OF TSPY PROMOTES TUMOR GROWTH IN NUDE MICE

The in vivo effects of TSPY expression were studied by tumorigenicity assays in nude mice. Both HeLa and NIH3T3 cell clones harboring either the bicistronic responder gene (TIG-TSPY) or the vector (TIG) alone were inoculated subcutaneously on the flanks of nude mice. Other animals were inoculated with the parental cells alone. They were fed either with or without doxycycline in their drinking water. Six animals were used for each criteria point. Tumor sizes were measured weekly for 7 weeks. Results showed that tumors grew significantly faster in nude mice inoculated with HeLa tet-off cells harboring a over-expressed TSPY gene than in those harboring the vector alone (including EGFP). Nude mice harboring the same HeLa cells whose TSPY gene is repressed by feeding doxycycline-containing drinking water showed the same rate of tumor growth as those in hosts inoculated with HeLa parental cells or those with vector alone. Significantly, the NIH3T3 cells are not tumor cells. Under normal conditions, no tumor should result from such inoculation. It was indeed the case for mice inoculated with the NIH3T3 parental cells or those harboring the vector alone. But, for those inoculated with NIH3T3 cells over-expressing a TSPY gene, small tumors were observed in 5 of the 6 mice in the group fed with normal drinking water. Again for those fed with doxycycline-containing water, no tumor was observed even they were inoculated with the same cells. Expression analysis indeed confirmed the expression of TSPY and EGFP in animals fed with normal drinking water and repression of the transgene in those fed with doxycycline-containing water. Results from this study suggest that over-expression of TSPY potentiates cell proliferation and promotes tumor growth in nude mice. Hence, TSPY is a putative proto-oncogene or oncogenic promoter gene on the human Y chromosome.

FUTURE DIRECTIONS

For the next funding period, we plan to continue the studies proposed in our original application. We believe that we have surveyed the expression of sufficient number of Y chromosome genes and have identified TSPY to be the most significant candidate gene identified so far that potentially may play a role in prostatic oncogenesis. Our work during the past funding period has detected various forms of TSPY transcripts from both normal and cancerous testes and prostates. Using the cDNAs for one of these variant transcripts, we have demonstrated that over-expression of its protein in cell cultures increases the cell proliferation activities of its hosts. When these cells are inoculated into nude mice, they either form tumors at a faster rate for tumorigenic cells (i.e. HeLa) or induce tumors for non-tumorigenic cells (i.e. NIH 3T3) in these immunodeficient hosts. Results from both studies corroborated with each other, supporting the hypothesis that TSPY is a cell cycle modulator and its aberrant expression potentiates cell proliferation both in vitro and in vivo.

Three important questions need to be addressed immediately. They constitute a modified series of studies initially proposed under Objective 2. First, do all the variant forms of TSPY transcripts, and hence their respective cDNAs, encode TSPY proteins that have the same function of potentiating cell proliferation and accelerating the rate of tumor formation in nude mice? To provide answers to this question, we plan to include the cDNAs for the variant TSPY transcripts in our in vitro cell proliferation and in vivo nude mouse tumor studies, as outline above. Second, what type(s) of TSPY transcripts are expressed in prostatic and/or testicular cancers? We plan to generate specific antibodies against the different variant TSPY proteins and use them in immunohistochemistry to analyze their respective expression patterns in prostate cancer samples. Successful identification of a cancer-associated isoform of TSPY will be significant for further development of diagnostic reagents for prostate cancer. Third, does over-expression of TSPY in the prostates of transgenic mice indeed result in prostatic oncogenesis. We will use transgenic mouse techniques to generate various transgenic mice that over express a TSPY transgene in their prostates. We will adopt two specific strategies for the transgenic studies. The first strategy involves a general over-expression of TSPY in transgenic mice, including both testis and prostate. In this strategy, a strong promoter, such as the β-action promoter, will be used to direct such a

general over-expression of the transgene. The second strategy utilizes the tet-off transgene regulation, as initially proposed and used in the cell culture and nude mouse experiments described above. To accomplish this objective, mice harboring a tissue-specific transactivator gene, such as those directed by the human PSA or rat probasin promoter, or a TSPY responder gene, i.e. TIG-TSPY, will be constructed. Bi-transgenic mice will be generated by crossing the transactivator and responder lines. The ability of an over-expression of the TSPY gene in inducing oncogenesis in the prostates will be evaluated using both histopathological and molecular analyses. Currently, we have constructed various transactivator and responder lines, including 5 transactivator lines directed by the rat probasin promoter, 4 transactivator lines directed by the human PSA promoter, and 5 founding lines for the responder gene, TIG-TSPY. These transactivator and responder lines are currently being crossed with each other and with reference lines, such as those harboring a tetO-Myc responder line, to generate bi-transgenic lines for further analysis.

Successfully implementation of these studies in the last phase of the currently funded project will provide important information regarding the oncogenic functions of the isoforms of the TSPY protein and the possible application of TSPY isoform detection as diagnostic procedures for prostate cancer.

KEY RESEARCH ACCOMPLISHMENTS

- Identify the TSPY gene to be a strong candidate oncogene or oncogenic promoter gene on the human Y chromosome.
- Detect the expression of multiple forms of variant TSPY transcripts in both normal and diseased prostate glands and testes.
- Demonstrate that over-expression of TSPY potentiates the proliferative activities in cultured cells.
- Demonstrate that HeLa cells, an oncogenic cell line, over-expressing TSPY and inoculated in nude mice have a faster rate of tumor formation than that in cells whose TSPY transgene is repressed.
- Demonstrate that NIH 3T3 cells, a non-oncogenic cell line, induces tumor formation when inoculated in nude mice if their TSPY transgene is over-expressed while no tumor is induced in the hosts if their TSPY transgene is repressed.

REPORTABLE OUTCOMES

- Demonstration of a preferential and elevated expression of TSPY in human prostate cancer and a hormonally induced rat model of prostate cancer. This study establishes the potential universal role of TSPY in male-specific cancers, emphasizing on prostate cancer.

  Lau Y-FC, Wang YZ and Komuves LG (2002). Expression of a gonadoblastoma candidate gene in human and an animal model of prostate cancer. In preparation

- Establishing the preliminary evidence for TSPY gene as a cell cycle regulator and promoter of cell proliferation in cultured cells and tumor formation in animals.

  Liu XX and Lau Y-FC (2001). Over-expression of the TSPY gene on the Y chromosome potentiates cell proliferation in vitro and in vivo. Am J Hum Genet 69 (supppl):245.

CONCLUSIONS

The initial premise of the present project is that the Y chromosome is a male-specific chromosome harboring genes important not only for a fetus to develop into a boy and then a man, but also physiology of being a male. Accordingly, misbehavior of some of these Y chromosome genes in the prostate, and other male-specific organs, contribute to the their oncogenesis. Through the support of the present grant, we completed an extensive survey on the expression of most of the 31 genes on the human Y chromosome on samples of prostatic and testicular cancers. This study identified the TSPY gene to be a strong candidate for an oncogene and/or tumor promoter gene on the human Y chromosome. We hypothesize that TSPY serves a vital function in directing the spermatogonial cells in entering meiosis, but when it is aberrantly expressed in the prostates, it either causes or potentiates oncogenesis in this organ. Our research demonstrates that over-expression of TSPY potentiates cell proliferation in vitro and promotes tumorigenesis in nude mice in vivo. These findings lay the rationale and foundation to use TSPY as a putative oncogene in establishing an animal model of prostate cancer using transgenic strategies.

SO WHAT

Results from the present investigation should be significant in our fight against prostate cancer. The identification of TSPY as a putative oncogene gene or tumor promoter gene has confirmed our postulation that misbehavior of one or a few of the genes on this male-specific chromosome contributes to the oncogenesis of prostatic cancer. Further elucidation of any correlation between the different TSPY isoforms and degrees of malignancy of prostate cancer will be significant for future development of new diagnostic procedures for prostate cancer. The establishment of a reversible or stage-arrested oncogenic model of prostate cancer in transgenic mice will be invaluable in understanding the disease mechanism and designing therapeutic strategies and treatments for this important disease in men.

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Cytogenetics and Cell Genetics

Expression of a candidate gene for the gonadoblastoma locus in gonadoblastoma and testicular seminoma

Y.-F.C. Lau,[a] P.M. Chou,[b] J.C. Iezzoni,[c] J.A. Alonzo[d] and L.G. Kömüves[a]

Departments of [a] Medicine and [d] Anatomic Pathology, VA Medical Center, University of California, San Francisco CA;
[b] Department of Pathology, Children's Memorial Hospital, Chicago IL;
[c] Department of Pathology, University of Virginia Health Science Center, Charlottesville VA (USA)

Dedicated to Professor Dr. Ulrich Wolf on the occasion of his retirement.

Abstract. The gonadoblastoma locus on the Y chromosome (GBY) predisposes the dysgenetic gonads of XY females to develop in situ tumors. It has been mapped to a critical interval on the short arm and adjacent centromeric region on the Y chromosome. Currently there are five functional genes identified on the GBY critical region, thereby providing likely candidates for this cancer predisposition locus. To evaluate the candidacy of one of these five genes, testis-specific protein Y-encoded (TSPY), as the gene for GBY, expression patterns of TSPY in four gonadoblastoma from three patients were analyzed by immunohistochemistry using a TSPY specific antibody. Results from this study showed that TSPY was preferentially expressed in tumor germ cells of all gonadoblastoma specimens. Additional study on two cases of testicular seminoma demonstrated that TSPY was also abundantly expressed in all stages of these germ cell tumors. The present observations suggest that TSPY may either be involved in the oncogenesis of or be a useful marker for both types of germ cell tumors.

Gonadoblastoma is a rare tumor that arises mostly in the dysgenetic gonads of phenotypic females who harbor some Y chromosome materials in their genome (Page, 1987). The tumor is composed of aggregates of primordial germ cells and sex cord elements resembling immature Sertoli and granulosa cells. These aggregates are surrounded by luteinized ovarian type stroma that may include Leydig or lutein-type cells (Scully, 1953, 1970). Gonadoblastoma has been considered to be an in situ germ cell malignancy from which invasive germ cell tumors can develop (Skakkebaek et al., 1987; Jorgensen et al., 1997; Looijenga and Oosterhuis, 1999; Heerbst et al., 1999).

The prevalence of gonadoblastoma among XY females had led David Page (1987) to hypothesize the presence of a locus, gonadoblastoma locus on the Y chromosome (GBY), that predisposes the dysgenetic gonads of these sex-reversed individuals to develop such in situ tumors. Page further predicted that the gene(s) encoded by the GBY locus has a normal function in the testis and acts as an oncogene only in the dysgenetic gonad. Using a panel of DNAs from XY females with gonadoblastoma, Page had initially mapped the GBY locus to deletion interval 3 on the short arm and intervals 4B-7 on the long arm of the Y chromosome. Additional studies further sublocalized this locus to a small region consisting of ~1–2 Mb of DNA in deletion intervals 3E–3G proximal to and 4B at the centromere and possibly 5E, a proximal interval on the long arm (Salo et al., 1995; Tsuchiya et al., 1995). Among the genes so far isolated from the human Y chromosome (Vogt et al., 1997; Lahn and Page, 1997; Lau and Zhang, 2000), there are five genes residing on this small region: amelogenin Y (AMELY), RNA binding

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Request reprints from Dr. Chris Lau, Division of Cell and Developmental Genetics, Department of Medicine, VA Medical Center, 111C5, 4150 Clement Street, San Francisco, CA 94121 (USA);
telephone: (415) 476-8839; fax: (415) 750-6633; e-mail: clau@itsa.ucsf.edu

motif Y (RBMY), protein kinase Y (PRKY), protein tyrosine phosphatase PTP-BL related Y (PRY), and testis-specific protein Y-encoded (TSPY). Hence, they are candidates for GBY. AMELY encodes an enamel protein in the tooth buds (Salido et al., 1992). RBMY is a repeated gene with a majority of its functional members residing at interval 6 on the long arm, outside the GBY critical region (Cooke, 1999). It expresses a protein with RNA binding motif in the nuclei of male germ cells. PRKY is a single copy gene coding for a putative cAMP-dependent serine/threonine protein kinase (Schiebel et al., 1997). Both RMBY and PRKY have a homologous gene, RBMX and PRKX respectively, on the X chromosome (Schiebel et al., 1997; Delbridge et al., 1999). PRY is a recently isolated repeated gene family coding for a protein related to the PTP-BL tyrosine phosphatase (Lahn and Page, 1997). Some copies of PRY are present outside the GBY region. TSPY is a repeated gene whose functional members are primarily located in two clusters, TSPYA and TSPYB, within interval 3 (Zhang et al., 1992; Conrad et al., 1996; Vogt et al., 1997) on the short arm and as a single-copy on the proximal region of the long arm (Ratti et al., 2000). TSPY shares tight homology to a family of cyclin B binding proteins, such as the SET oncoprotein and the nucleosome assembly protein (NAP-1) (Tsuchiya et al., 1995; Schnieders et al., 1996), and has been postulated to play a role in directing the spermatogonial cells to enter meiosis (Schnieders et al., 1996; Vogel et al., 1998). Other cyclin B binding proteins have been demonstrated to be involved in the mitotic process, cell proliferation and/or carcinogenesis (von Lindern et al., 1992; Adachi et al., 1994; Altman and Kellogg, 1997; Carlson et al., 1998; Shin et al., 1999). Hence, aberrant or inappropriate expression of TSPY in dysgenetic gonads may play a role in the etiology of gonadoblastoma.

To evaluate the candidacy of TSPY as the gene(s) for GBY, we have performed detailed expression analysis of TSPY in gonadoblastoma and testicular seminoma, or germ cell tumor, using immunohistochemical techniques. Our results demonstrate that TSPY is preferentially expressed in the germ cells of the tumor aggregates in gonadoblastoma and tumor cells at different stages of testicular seminoma. Its expression pattern is very similar to those of cyclin B1 and another cell proliferative marker, the proliferating cell nuclear antigen (PCNA). These findings, hence, support the postulation that TSPY is a significant candidate for GBY, and suggest a possible role of TSPY in the multi-step carcinogenesis of testicular seminoma.

Materials and methods

Patients

Tissue sections were obtained from archival formalin-fixed and paraffin-embedded tumor specimens. All three gonadoblastoma patients had previously been described (Iezzoni et al., 1997; Hussong et al., 1997). At the time of biopsies, Patient #1 was a 15-year old phenotypic female with a unilateral gonadoblastoma at the left gonad. Patient #2 was a 20-year old phenotypic female with bilateral gonadoblastoma. Both patients #1 and 2 have a 45,X/46,XY mosaic karyotype. Chromosome painting analysis on tissue sections of these tumors showed that most tumor cells harbored a Y chromosome while the stroma showed reduced numbers of cells harboring this chromosome (Iezzoni et al., 1997). Patient #3 was a 15-year old phenotypic female with a 46,XY karyotype. She developed a tumor mass on the left and a streak gonad on the right (Hussong et al., 1997).

Two testicular seminoma specimens were obtained from archival samples at the Anatomic Pathology Section, VA Medical Center, San Francisco. At the time of orchiectomy, Patient #1 was a 49-year old male with a tumor mass confined only to the left testicle. Pathological examination revealed a classical seminoma. Patient #2 was a 49-year old male with an advanced and mixed germ cell tumor composed of seminoma, embryonal carcinoma and yolk sac tumor.

Generation of a specific antibody against TSPY

The entire open reading frame of the human TSPY cDNA (Zhang et al., 1992) was subcloned in-frame in the *Eco*R1 site of the expression vector, pAR(ΔR1) (Blanar and Rutter, 1992), a derivative of the pET3a vector. Recombinant TSPY protein was synthesized in bacterial host, BL21DE3 (pLysS) and purified by preparative SDS-PAGE from total lysates of induced bacterial culture. A polyclonal antiserum was generated by repeat immunizations of a New Zealand white rabbit using the service of a commercial vendor (Vancouver Biotechnology, Vancouver, Canada). The specificity of the antiserum was initially assayed by Western blotting against recombinant TSPY protein. The specificity of this antibody to TSPY was further confirmed by both Western blotting and immunocytochemical staining of HeLa cells expressing at high levels a transfected human TSPY gene (Lau, unpublished observations). A polyclonal antibody against the proliferative cell nuclear antigen (PCNA) was purchased from Dako Laboratory, Inc. (Carpinteria, CA). A polyclonal antibody against the human cyclin B1 (synthesized with a bacculuvirus vector in insect cells) was a gift from Catherine Takizawa and David Morgan, Department of Physiology, UCSF. Both antibodies had previously been demonstrated to be specific for the respective antigens in Western blotting and immunostaining studies (Jin et al., 1998; Takizawa et al., 1999; Kömüves et al., 1999).

Immunohistochemistry of tumor tissue sections

Five-micron sections were obtained from archival materials according to established procedure. Immunohistochemical staining was performed as previously described (Kömüves et al., 1999). Heat-induced antigen retrieval pretreatment was utilized in procedures with PCNA and cyclin B1 antibodies. Immunostaining was conducted in a Tris buffer, pH 7.6, containing 4% of bovine serum albumin, 1% gelatin, 0.1% Tween 20, and 500 mM NaCl. The primary antisera were used at 1:500 to 1:1000 dilution ratios. The binding of the primary antibody was detected by reaction with affinity-purified biotinylated goat anti-rabbit IgG, and visualized by either ABC-peroxidase or ABC-alkaline phosphatase reagents. Commercial substrate kits (Vector Laboratories, Burlingame, CA) were used for the enzymatic detections. For the brown and brick-red signals, the DAB and VECTOR NovaRED kits were used respectively with the ABC-peroxidase reagents. For the red signal, the VECTOR RED kit was used with the ABC-alkaline phosphatase reagents. All signals were dependent on the bindings of the respective primary antibodies and were independent of the substrate kits used. After the immunostaining, the sections were counter-stained with hematoxylin. Omitting the primary antibody in the procedure resulted in no signals. Preabsorption of the TSPY antiserum with excess recombinant TSPY protein abolished the staining. The sections were examined and recorded with a Zeiss Axiophot microscope.

Results

Preferential expression of TSPY in tumor germ cells of gonadoblastoma

Histological analysis of the gonadoblastoma specimens from all three patients showed characteristic aggregates of primordial germ cells and sex chord elements. Immunostaining of tissue sections of all four tumors (two patients with unilateral and one patient with bilateral tumors) showed positive staining of cancerous germ cells within these tumor aggregates of all three patients (e.g. Fig. 1A). In particular, the tumor of patient #3 was organized in smaller aggregates with less sex chord elements (Fig. 1B, C), partially resembling the morphology of some forms of germ cell tumors in the testis (e.g. Fig. 1L, M).

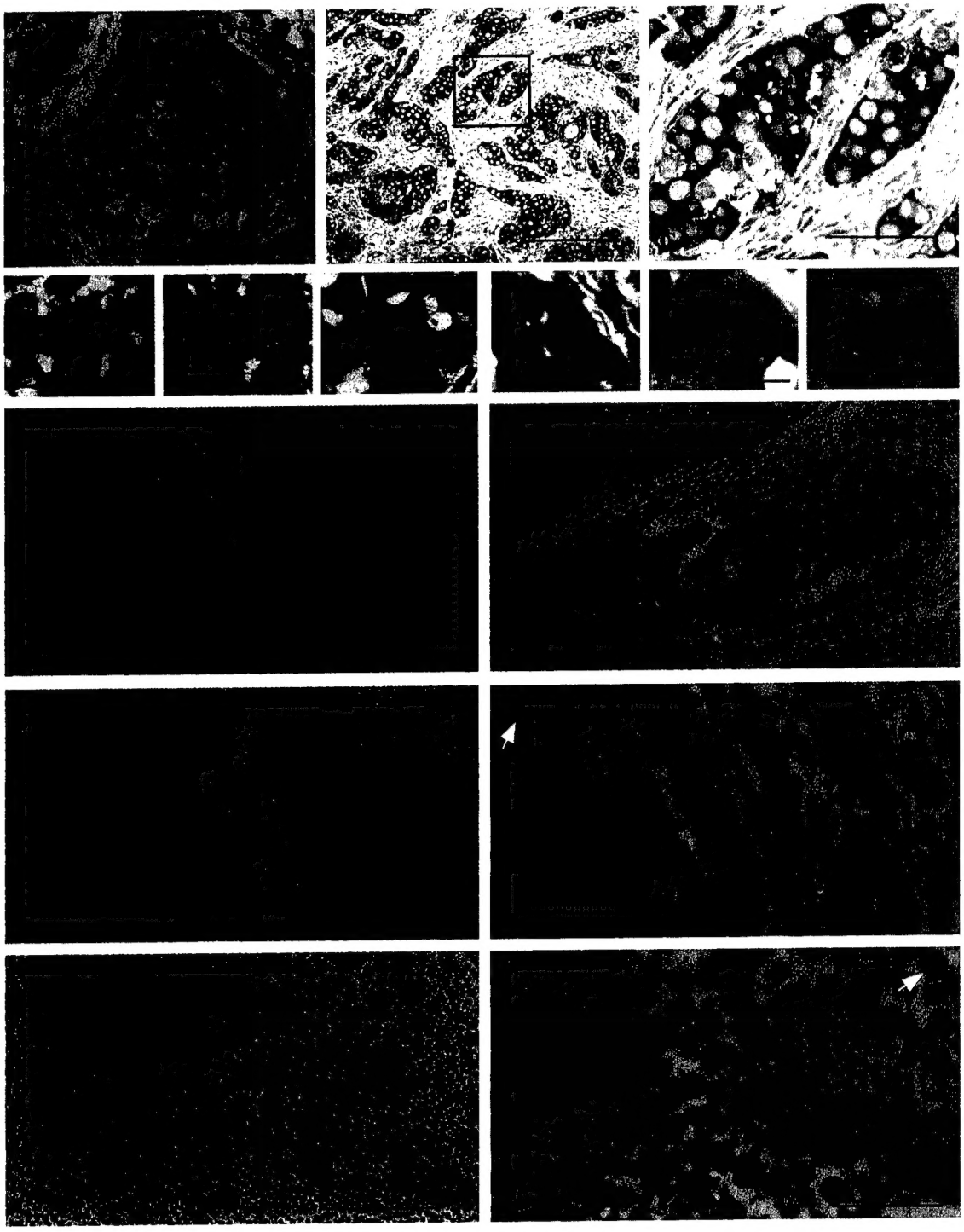

Fig. 1. Immunohistochemical localization of TSPY in gonadoblastoma and testicular seminoma. (**A**) TSPY was primarily located in the germ cells of tumor aggregates of all three gonadoblastoma patients. This figure illustrates an example of immunostaining on sections from Patient #1. (**B**) Patient #3 harbored a gonadoblastoma with less organized aggregates. (**C**) An enlargement of boxed area in **B**, showing prominent cytoplasmic locations of the TSPY protein. (**D–I**) Examples of mitotic cells within the gonadoblastoma from patient #3. Cells in **D–G** were stained with TSPY antibody; **H** with PCNA antibody and **I** a control without primary antibody reaction. (**J–O**) Immunostaining of TSPY on tumor sections from seminoma at early (**J**), intermediate (**K**) and late (**L–O**) stages of the testicular cancer. Yellow arrows in **J** point to possible localized tumor growth areas. Blue arrow in **L** indicates a tubule being abandoned by the invasive growth of the tumor (boxed area enlarged in **M**). **N** Shows an advanced tumor area consisting of large mass of tumor cells and highly undifferentiated and loosely associated embryonal cells (boxed area enlarged in **O**). White arrows in **M** and **O** point to mitotic tumor cells. Positive signals are brick red in **A**; chocolate in **B** and **C**; brown in **D–H**; and red in **J–O**. Bars indicate 50 μm in **A**, **C**, **M** and **O**; 200 μm in **B**, **J**, **L**, and **N**; and 10 μm in **D–I**.

The TSPY protein was prominently localized primarily in the cytoplasm of these large cells (Fig. 1C). In all cases, the sex cord elements and the stroma showed very little reactive signals (Fig. 1A, B). Mitotic cells were readily observed on specimens from this patient (Fig. 1D–I). Omission of the primary antibody or pre-absorption of the antiserum with excess recombinant TSPY protein abolished or greatly reduced the reactive staining of the germ cells in these procedures (e.g. Fig. 1I). Analysis of parallel sections with PCNA and cyclin B1 antibodies showed similar staining patterns as those with the TSPY antibody (data not shown). The signals were primarily located on the nuclei for PCNA while those for cyclin B1 seemed to be associated with both nuclei and cytoplasm of the germ cells. The general staining patterns indicated that TSPY, PCNA and cyclin B1 were co-expressed in the same tumor cells.

TSPY expression in the tumor cells at various stages of testicular seminoma

It has been argued that gonadoblastoma is a precursor form of more aggressive germ cell tumors (Skakkebaek et al., 1987; Jorgensen et al., 1997; Looijenga and Oosterhuis, 1999; Herbst et al., 1999). If TSPY is indeed the candidate for GBY, it would potentially participate in the oncogenic process of other germ cell tumors. Previously, Schneiders and colleagues (1996) had indeed demonstrated an up-regulation of TSPY expression in in-situ carcinoma of the testis, a presumed precursor of germ cell tumors or seminoma. To address the question of whether TSPY may also play a role in more advanced testicular cancer, we have extended our study to include two cases of seminoma using the same immunohistochemical staining technique and specific antibodies against TSPY, PCNA and cyclin B1. They, together, showed various morphological forms potentially representing the different oncogenic stages of these tumors. At the early stages, spermatogenesis might have ceased, thereby depleting normal meiotic cells within the seminiferous tubules. In-situ carcinoma could also occur as a precursor during this initial carcinogenic period (Schnieders et al., 1996). The germ cell-depleted and epithelium-like tubules consisted of a single-layer of cells that might develop into localized multi-layer tumors at various peripheral segments (Fig. 1J, yellow arrows). This transformation progressed until most of the epithelia were lined with multiple layers of tumor germ cells (Fig. 1K). Invasive aggregates of cancerous germ cells eventually evolved from such tubular tumors, abandoning the original tubules (Fig. 1L, blue arrow). In the advanced stages, these aggregates could form a large tumor mass covering a sizable portion of the testis (Fig. 1L, N). Occasionally highly undifferentiated and loosely associated embryonal cells were observed in the late stages of these tumors (Fig. 1O). Presumably, these single tumor cells might have acquired some metastatic properties/potential. Significant expression of the TSPY protein was detected in the tumor germ cells at all stages of these testicular cancers (Fig. 1J–O). Similar to the expression pattern in gonadoblastoma, TSPY was primarily located on the cytoplasm of the tumor cells (Fig. 1M, O). Occasionally, nuclear locations of TSPY were also detected in a few cells. Under each microscopic view, a significant number of mitotic cells could easily be identified (white arrows, Fig. 1M, O). Similar to those in gonadoblastoma, most mitotic cells were stained positively with the TSPY antibody. TSPY expression was at a reduced or insignificant level in the interstitial regions of these testicular cancers.

Immunohistochemical staining of parallel tissue sections with PCNA and cyclin B1 antisera demonstrated similar expression patterns as that of TSPY for both antigens in testicular seminoma (data not shown). Again, PCNA showed a mostly nuclear location while cyclin B1 showed a nuclear and cytoplasmic staining pattern. These observations suggested a possible co-expression of these three proteins in the same tumor germ cells.

Discussion

The mapping of the GBY locus within a small region of the human Y chromosome suggests the existence of a proto-oncogene on this chromosome that predisposes the dysgenetic gonads of XY females to malignancy (Page, 1987; Salo et al., 1995; Tsuchiya et al., 1995). The identification of candidate genes for GBY will not only provide information on the molecular etiology of gonadoblastoma but will also shed light on the contribution of this chromosome to the carcinogenic processes of other male-specific cancers, such as testicular seminoma, germ cell tumors and prostate cancer (Looijenga and Oosterhuis, 1999; Lau, 1999; Lau and Zhang, 2000). Recent studies on TSPY have positioned it to be the most viable candidate for GBY within the critical region on the Y chromosome. First, it is present in the DNAs of gonadoblastoma patients (Salo et al., 1995; Tsuchiya et al., 1995). Its transcripts had been detected by RT-PCR technique in the corresponding tumor tissues (Tsuchiya et al., 1995) and recently by immunostaining in a single case of gonadoblastoma (Hildenbrand et al., 1999). Second, immunostaining studies had demonstrated its expression in spermatogonia in normal testis (Schnieders et al., 1996), suggesting that TSPY may serve a normal function of directing the spermatogonial cells to enter meiosis, a condition for the GBY gene(s) initially postulated by David Page (1987). Third, the TSPY protein is highly homologous to a family of cyclin B binding proteins, including NAP-1 and SET oncoprotein (Tsuchiya et al., 1995; Schnieders et al., 1996), suggesting that it may bind to this mitotic cyclin and be involved in cell cycle regulation and/or cell proliferation (Shin et al., 1999). Results from the present study demonstrate that TSPY is preferentially expressed in the proliferating germ cells within the tumor aggregates in all four samples from three gonadoblastoma patients, further supporting the candidacy of TSPY for GBY.

Although TSPY protein has previously been detected in some forms of testicular tumors, including in-situ carcinoma (Schnieders et al., 1996), our study on the seminoma specimens has clearly demonstrated the high levels of expression of this GBY candidate gene in all stages of these advanced germ cell tumors. Hence, these results, together with those observed by others (Schnieders et al., 1996), establish a direct relationship between the aberrant TSPY expression and the oncogenic process of testicular cancer. Numerous studies have demonstrated that cyclin B binding proteins, such as SET, are either involved in oncogenesis of acute leukemia, Wilm's tumor or modulation

of cell proliferation (von Lindern et al., 1992; Adachi et al., 1994; Carlson et al., 1998; Shin et al., 1999). Although the interactions between TSPY and cyclin B have yet to be demonstrated experimentally, the co-expression of these two molecules on the same tumor cells has raised the possibility that they may indeed interact in vivo. The present study has provided evidence supporting the hypothesis that aberrant expression of TSPY may lead to abnormal cell proliferation and tumor formation (Lau, 1999). Hence, TSPY is not only a key candidate for GBY but may also contribute to the oncogenesis of testicular seminoma.

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Note added in proof

Recently Stuppia et al. (2000) have demonstrated by cloning and sequence analysis that the PRY gene spans 25 kb in size and contains 5 exons. The functional copies of PRY are located in interval 6 on Yq while those on Yp retain only exon 1 and 2 and are likely non-functional.

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Overexpression of cyclins B observed in human tumors alters chromosomal segregation. *J.M. Flaman[1], N. Sarafan-Vasseur[1], J. Bourguignon[1], F. Le Pessot[2], A. Lamy[1], R. Sesboüé[1], C. Bastard[1], P. Hieter[3], T. Frébourg[1].* 1) INSERM-EMI 9906, IFRMP, Faculté de Médecine, Rouen, France; 2) Anatomie et Cytologie Pathologiques, CHU de Rouen; 3) CMMT, Vancouver, Canada.

The molecular basis of chromosomal instability (CIN), which is observed in most of the malignant tumors, remains to be characterized. To identify genes which overexpression results in CIN, we developed a biological approach based on the use of a yeast indicator strain for CIN. In this strain, the ade2- mutation, resulting into the accumulation of a red pigment, has been suppressed by an ochre suppressing tRNA, carried by a non essential artificial chromosomal fragment, and this strain is therefore spontaneously white. Induction of CIN in this strain will result into the loss, during the mitosis, of the chromosomal fragment and therefore into the appearance of red sectors within the white colonies. Screening into this CIN indicator strain of a yeast genomic library led us to identify, among the clones generating 100% of sectoring colonies, Clb5, one of six B-type cyclins identified in yeast. Overexpression of cyclin B2 and cyclin B1; the two human homologs of Clb5, into the CIN indicator strain resulted also into a sectoring phenotype and induced, like overexpression of Clb5, an abnormal sensitivity to benomyl, indicating that overexpression of B type cyclins alters the spindle checkpoint. Using multiplex real-time quantitative RT-PCR, we analyzed cyclins B1 and B2 mRNA expression in a serie of 58 primary colorectal cancers. In 10% of the tumors, we observed a 10 fold increase of cyclin B2 mRNA, in comparison to normal colorectal mucosa. Although we detected no overexpression of cyclin B1 mRNA, immunohistochemical staining revealed a high level of cyclin B1 expression in 12 % of the tumors. These results show that overexpression of cyclins B is observed in human cancers and could contribute, through an alteration of the spindle checkpoint, to chromosomal instability.

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Paternal uniparental disomy 11p15, hemihyperplasia and hepatoblastoma. *L. Celle[1], K.L. Russell[1], D.J. Zand[1], A.T. Meadows[2], J. Pressey[2], D. von Allmen[3], R. Weksberg[4], E.H. Zackai[1].* 1) Division of Human Genetics and Molecular Biology; 2) Division of Oncology; 3) Department of Pediatric General and Thoracic Surgery, The Children's Hospital of Philadelphia, Philadelphia, PA; 4) Department of Genetics, Hospital for Sick Children, Toronto, Canada.

Hepatoblastoma is a rare embryonal tumor occurring most frequently before the age of 3 years. Patients with Beckwith-Wiedemann syndrome (BWS), isolated hemihyperplasia and the combination of both have an increased risk of neoplasia with Wilms' tumor being the most common, followed by adrenocortical carcinoma and hepatoblastoma. Patients with BWS and some with isolated hemihyperplasia have been shown to have deregulation of normal imprinted expression on chromosome 11p15. Here we present a child with hemihyperplasia with paternal UPD 11p15 who, after tumor surveillance, was found to have an elevated AFP and subsequent diagnosis of hepatoblastoma. A 4.5-month old boy was referred to Genetics for hemihyperplasia. He was a 9lb 1oz product of a FT pregnancy without neonatal hypoglycemia. On physical exam, there was marked hemihyperplasia of the right arm and leg, with a 1cm difference in the length of the humeri and tibiae. There was also an enlargement of the right side of the tongue. There was no umbilical defect and no abnormal ear creases. Molecular genetic studies showed paternal UPD of chromosome 11p15. He was followed with abdominal ultrasounds and alpha-feto protein (AFP) levels. At 5months, the AFP level was noted to be 325.8ng/ml (normal 0.6-28.3), fell during the next several months, and rose again at 10months. Although the ultrasound at that time was normal, a CT scan and MRI with gadolinium enhancement revealed a 2 x 1.7cm lesion in the right lobe of the liver. Following complete excision, pathology revealed hepatoblastoma, mixed histology. This report emphasizes that children with hemihyperplasia should undergo molecular testing. They should also have careful tumor surveillance, especially those with paternal UPD 11p15.

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Chromosomal amplifications and deletions determined by comparative genomic hybridization in follicular thyroid adenomas and carcinomas - similar patterns of chromosomal aberrations in a subset of thyroid carcinomas. *D.J. Marsh[1], G. Theodosopoulos[1], L. Delbridge[2], B.G. Robinson[1,3].* 1) Kolling Institute of Medical Research and University of Sydney, NSW, Australia; 2) Department of Surgery, Royal North Shore Hospital, NSW, Australia; 3) Department of Medicine, University of Sydney, NSW, Australia.

Carcinoma of the thyroid is the most frequently diagnosed endocrine malignancy. Malignant lesions of the follicular cells are comprised of the differentiated tumors follicular thyroid carcinoma (FTC) and papillary thyroid carcinoma, whilst anaplastic thyroid carcinoma is an undifferentiated malignancy. Follicular thyroid adenomas (FTAs) are benign tumors. We used comparative genomic hybridization (CGH) to elucidate regions of chromosomal gains and losses in both FTAs and FTCs. Seventy-six % (13 of 17) of FTCs showed CGH changes, however only 24 % (5 of 21) of FTAs showed changes. The number of CGH changes varied markedly, with FTAs showing between 1 - 8 (mean =3.6) CGH changes, and FTCs showing 3 - 23 (mean = 11.5). Of the changes observed in FTAs, none were observed more than once. However, certain "hot-spots" were evident in the FTCs including gain of chromosomes 7, 5 and 12, as well as loss of chromosomes 1, 8 and 11. A number of chromosomal alterations were found in both FTAs and FTCs, including loss of 3p, 4, 8, 9p and X, as well as gains of 4q, 5p, 7, 8, 9, 12 and 17. Gain of 1p and loss of 5q were unique to FTAs. Of considerable interest, 6 of the FTCs had between 14 - 23 (mean = 20) CGH changes, and these changes appeared to be largely consistent between the tumors, including loss of chromosome 1, 2, 3, 6, 8, 11 and X, as well as gains of chromosomes 5, 7, 12, 19 and 22. This data is suggestive that activation of a mutator phenotype occurs at some point in a progression pathway during thyroid tumorigenesis and in at least a subset of tumors, leads to a non-random pattern of chromosomal gains and losses. Alternative patterns of chromosomal gains and losses may not be conducive to survival of the malignant cell. It is possible that such a mutator phenotype involves the alteration of a gene(s) functioning to regulate mitosis.

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Over-Expression of the TSPY Gene on the Y Chromosome Potentiates Cell Proliferation in vitro and in vivo. *X.X. Liu, Y.F.C. Lau.* Department of Medicine, University of California, San Francisco, CA.

The testis-specific protein Y-encoded (TSPY) gene is a repeated gene on the human Y chromosome. It encodes a protein homologous to a family of cyclin B binding proteins and is postulated to serve a normal function in directing the spermatogonial cells to enter meiosis in the testis. Based on its chromosomal location, it has been considered to be a candidate for the gonadoblastoma (GBY) locus on the Y chromosome. Expression studies indeed demonstrated a high level expression of this GBY candidate gene in gonadoblastoma and testicular seminoma. We hypothesized that aberrant expression of TSPY in cells incapable of entering male meiosis is responsible for its oncogenic activities. To test this hypothesis, we used the tetracycline (Tet-off) regulation system to manipulate the expression of transfected TSPY gene in HeLa or NIH3T3 cells. Cells overexpressing TSPY exhibited a 30-45% higher proliferative activities than those under repressed conditions or transfected with the vector alone. This increase in proliferative activities paralleled with an elevated rate of colony formation in transfected cell population cultured in normal media than that cultured in media containing doxycyline. Subcutaneous inoculation of stably transfected HeLa cells in immunodeficient nude mice showed a faster rate of tumor formation in hosts fed with normal drinking water than that in hosts fed with doxycycline-containing water. Significantly, NIH3T3 cells over-expressing the stably transfected TSPY gene formed tumors in nude mice fed with normal drinking water. However, the same transformant cells inoculated in hosts fed with doxycycline-containing water did not produce any tumor. Results from this study support the hypothesis that over-expression of the Y-located TSPY gene potentiates cell proliferation in vitro and in vivo, thereby signifying TSPY as a putative proto-oncogene on the human Y chromosome.

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Loss of heterozygosity of the CUTL1 gene in uterine leiomyomata. *S.M. Zeng, B.J. Van Voorhis, J. Yankowitz.* Dept of OB/GYN, Univ Iowa Hosps & Clinics, Iowa City, IA.

Uterine leiomyomata or fibroids are common benign smooth muscle tumors. These tumors are the single largest cause of hysterectomy in the United States, accounting for approximately one in three hysterectomies or 200,000 procedures a year in the United States. The underlying cause of fibroids is still obscure. Recent molecular genetic and cytogenetic evidence indicates several gross and microscopic genetic alterations involving five main subgroups; del(7q), 6p rearrangements, del(13q), t(12,14), and trisomy 12. The gross and microscopic deletions at 7q may involve the human Cut-like homeobox gene, CUTL1, located at 7q22. We investigated loss of heterozygosity (LOH) of this gene in patients with uterine leiomyoma.

Samples of uterine fibroids and grossly normal myometrium were obtained from women having myomectomies or hysterectomies. Tissues were digested and DNA extracted by standard techniques. DNA samples were amplified using different sets of primers specific for three microsatellite markers inside CUTL1, D7S515, D7S518 and D7S666. PCR products were run on a 6% denaturing polyacrylamide gel. LOH was evaluated by comparing allele bands between tumor and normal uterine tissue from the same patient.

Twenty patients with both fibroid tissue and normal surrounding tissue were evaluated. A CUTL1 deletion was found in three fibroid samples (15%). In one of these patients LOH was also present in one of two "normal" surrounding tissue samples. In one patient with LOH in one large fibroid another small fibroid did not show LOH for the CUTL1 markers.

Our results agree with previous findings from cytogenetic and molecular genetic studies supporting the hypothesis that CUTL1 gene may be a tumor suppressor and whose deletion or loss may play a role in the pathogenesis of uterine leiomyoma. Additional studies are needed to elucidate the relationship between DNA alterations, potential other factors, and fibroid development.

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Identification of a minimal overlapping amplified region at 19q13.1-q13.2 in four ovarian cancer cell lines. *T.C.M. Tang, J.S.T. Sham, X.Y. Guan.* Department of Clinical Oncology, The University of Hong Kong, Hong Kong, People's Republic of China.

High-level amplification of DNA sequence at 19q13.1-q13.2 in ovarian cancer has been frequently detected by using chromosome microdissection and comparative genomic hybridization (CGH). This strongly suggests that 19q13.1-q13.2 contains a putative oncogene(s) which plays an important role in the development or progression of ovarian cancer. In order to narrow down the amplified region at 19q13.1-q13.2 (about 39cM), four ovarian cancer cell lines which have been confirmed containing amplicon at 19q13.1-q13.2 were studied for identifying a minimal overlapping amplified region (MAR). Fluorescence in situ hybridization (FISH) with cosmid clones and Southern blot analysis with cDNA probes in average genomic distance at 1 cM (from D19S425 to D19S418) were performed. At least two MARs have been identified at 19q13.12 (about 200 kb) and 19q13.13 (about 1.2 Mb), respectively. Amplification and overexpression of several candidate oncogenes within the two amplicons have been studied by Southern and Northern blot analysis in four ovarian cancer cell lines. Further study of genes within these two amplicons may lead to the isolation of the oncogene(s) which is the biological target of amplification events in ovarian cancer.